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Plasma neuronal exosomes serve as biomarkers of cognitive impairment in HIV infection and Alzheimer's Disease

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Abstract

Fluid biomarkers for cognitive impairment have the advantage of being relatively noninvasive and capable of monitoring neuronal and other brain cell health in real time. Biomarkers can predict the onset of dementing illness, but also correlate with cognition in a dynamic way allowing us to follow treatment responses and determine brain recovery. Chronic HIV infection causes cognitive impairment in a subset of individuals suggesting "premature aging." Exosomes are small extracellular vesicles that are shed from all cells. They are important in normal cell to cell communication as they contain cellular proteins, mRNA transcripts and miRNAs. Exosome cargo varies depending on the health of the cell and pathological state; specific proteins/mRNAs and/or miRNAs are present and may serve as biomarkers. Exosomes of variable cellular origin can be isolated from peripheral blood by various methods. Neuron-derived exosomes (NDEs) can be isolated using a precipitation/immunoaffinity approach using antibodies against neuronal cell adhesion molecule L1CAM and the contents queried for central nervous system (CNS) disorders including HIV-associated neurological disorders (HAND) and Alzheimer's disease (AD). As these studies are recent, numerous questions arise including which neuronal proteins are in NDEs and whether their contents differ in different CNS pathologies or with age. In addition, can the NDE cargo predict as well as diagnose cognitive impairment and could exosomal contents be used as therapeutic biomarkers, or theramarkers, of neuronal recovery from effective treatment? This mini-review will show some new data and review recent studies on NDE from individuals with HIV infection and AD.

Keywords

Exosomes; neurons; biomarker; HIV; Alzheimer's; dementia

HIV-associated neurocognitive disorders (HAND) are pathologies seen in a subset of individuals with chronic HIV infection. They belong to the spectrum of neurodegenerative

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The authors declare that there is no conflict of interests.

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diseases that result in death or dysfunction of neurons with similarities to Alzheimer disease (AD) but also distinctive differences (Reviewed (Canet *et al*, 2018)). Both disorders are difficult to diagnose without neuropsychological testing and both need new biomarkers to judge progression as well as recovery with treatment. Both disorders involve neuroinflammation and several common targets. AD is associated with aging and HIV is thought to initiate premature aging. In HIV infection, amyloid beta (Aβ), which is deposited in "plaques" in AD, is soluble and its relevance to HIV-associated cognitive impairment is controversial (Achim *et al*, 2009; Rempel and Pulliam, 2005). Aβ deposition is required for AD pathological diagnosis, but is not necessarily causative (Barage and Sonawane, 2015; Hardy and Selkoe, 2002; Morris *et al*, 2014). Neurofilament light (NF-L) is a surrogate marker in plasma and cerebrospinal fluid (CSF) for neurodegeneration (Abu-Rumeileh *et al*, 2018; Mattsson *et al*, 2017) but continues to be a controversial biomarker for both HAND and AD (Gisslen *et al*, 2016; Kovacs *et al*, 2017; Norgren *et al*, 2003; Rolstad *et al*, 2015; Yilmaz *et al*, 2017). Blood biomarkers are needed to advance both HAND and AD fields, as blood draws are less costly than neuroimaging and are minimally invasive compared to lumbar punctures required for CSF acquisition.

Extracellular vesicles (EVs) are nanoscale membranous vesicles shed from all cells including those of the central nervous system (CNS) and found in all biofluids; they are divided into exosomes (30 – 150 nm) originating from late endosomes/multivesicular bodies and microvesicles (150 – 1000 nm) produced through budding of the plasma membrane. Both types of vesicles are implicated in the pathogenesis of neurodegenerative diseases and may provide biomarkers (Bellingham *et al*, 2012). In this report we call the vesicles exosomes, since they are the predominant vesicles in our preparations. They are involved in cell to cell communication in normal homeostasis and can be carriers of toxic proteins (Aβ, tau) (Sardar Sinha *et al*, 2018) shed by cells as waste or actively secreted in a degenerative process (Review (Gupta and Pulliam, 2014)). The idea that exosomes originating from a specific cell can be recovered in the plasma using cellular surface markers of interest is intriguing. Neuron derived exosomes (NDEs) were first described in 2015 and isolated using antibodies against neural cell adhesion molecules NCAM or L1CAM, after total plasma exosome isolation (Fiandaca *et al*, 2015). Characterization of NDEs follows guidelines endorsed by the International Society for Extracellular Vesicles and includes Nanoparticle Tracking Analysis (NTA) to determine EV concentration and average diameter; Western Blots for EV markers; ELISAs for neuronal proteins and transmission EM for visualization (Sun *et al*, 2017; Tang *et al*, 2016).

This innovative isolation of an exosome sub-population has generated interest in using NDE as biomarkers for neurodegenerative diseases like AD, HAND, traumatic brain injury, posttraumatic stress disorder and more (Reviews, (Agoston *et al*, 2017; Gupta and Pulliam, 2014; Hu *et al*, 2016; Karnati *et al*, 2018; Osier *et al*, 2018)). Several biomarkers from plasma NDEs were recently reported by the Pulliam lab to be elevated in general cognitive impairment (Sun *et al*, 2017). We review our collective data here on HAND and AD and add to the characterization of plasma NDEs as exciting biomarkers of neurodegeneration.

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NDEs in HIV-associated neurological disorders (HAND)

Neuronal exosomes are enriched by antibody to L1CAM after total exosome isolation from plasma using ExoQuick or ExoQuick-Ultra, as previously described (Mustapic *et al*, 2017; Sun *et al*, 2017). The average diameters and concentrations of total exosomes and NDEs were determined using a nanoparticle tracking system NanoSight® LM10. There was no difference in average diameters between total exosomes from control subjects and those infected with HIV; however, there were more total exosomes from HIV-infected subjects (Sun *et al*, 2017). Electron microscopy of exosomes showed a uniform round shape with delineation of a darker membranous periphery and a central clearing (Tang *et al*, 2016). There was no difference in numbers of NDEs between the HIV-infected and control groups. We further characterized exosomes from HIV-infected individuals and controls by quantification of tetraspanins CD63, CD81 and exosomal markers ALIX and EpCAM. Both CD63 and CD81 had different levels in NDEs from controls compared to HIV infection (Fig. 1A) (Sun *et al*, 2017). We recently evaluated EpCAM and ALIX levels on NDE. EpCAM levels were below the level of detection by ELISA (data not shown) but ALIX showed not only good measurable concentrations but comparable levels between control and HIV NDEs (Fig. 1A). Normalizing to counts may not be practical when studying large numbers of small volume specimens. ALIX may serve as a good reproducible biomarker for normalization when exosome counts are impractical or counting mechanisms unavailable.

L1CAM is expressed on hematopoietic and transformed epithelial cells and highly enriched on neurons. To confirm neuronal enrichment of the L1CAM-isolated exosomes, neuronal NF-L and synaptophysin (SYP) were assayed and shown to be significantly enriched in NDEs (Sun *et al*, 2017).

For initial studies on HIV NDEs, neuronal targets were chosen based on the literature and association with HIV cognitive impairment. Aβ is elevated in brains of HIV-infected individuals (Achim *et al*, 2009; Green *et al*, 2005; Rempel and Pulliam, 2005) as well as in human neuronal cells *in vitro* when exposed to HIV Tat (Daily *et al*, 2006; Rempel and Pulliam, 2005). While our initial studies showed that Aβ was increased in brains at autopsy of HIV-infected individuals, Aβ was found in soluble forms and not deposited in plaques (Rempel and Pulliam, 2005). Now with people living into old age with HIV infection and on antiretroviral therapy, the significance of Aβ should be re-examined. We looked at Aβ in NDEs from HIV subjects and found it to be significantly increased although there was a wide range of values (Fig. 1B) (Sun *et al*, 2017).

Neurofilament light is an axonal degeneration marker associated with neuronal damage that is significantly elevated in CSF in HIV dementia (Peterson *et al*, 2014). With the advent of antiretroviral therapy, this biomarker decreases in both CSF and plasma and unfortunately has not been a reliable indicator of HAND (Gisslen *et al*, 2016); reviewed in (Anderson *et al*, 2018). When looking at NDEs, NF-L was notably elevated in impaired individuals demonstrating the specificity of neuronal origin and injury compared to plasma or CSF (Fig. 1B).

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As a general marker of neuronal injury and neurocognitive impairment, high mobility group box 1 (HMGB1) was measured in NDEs (Fig. 1B). An increase in HMGB1 in NDE from impaired individuals suggests that neurons are damaged and are releasing toxic products via exosomes. Importantly in these studies, there was no difference in these 3 NDE targets between HIV-infected and uninfected subjects but rather impaired versus unimpaired individuals, suggesting specificity to neurodegenerative processes.

Age-related fluctuations in NDEs from controls have not been extensively studied. We began to look at this with several targets. There was no significant positive or negative correlation between normal aging and NDE proteins HMGB1, NF-L or Aβ (Fig. 1C) (Sun *et al*, 2017). However, there was a significant negative correlation of HMGB1 and borderline significant correlation of NF-L from NDEs of HIV-infected subjects with aging (Fig. 1C) and no significant change in Aβ (data not shown). This may reflect a loss of neurons and brain atrophy with aging in HIV infection. The controls in our study clustered around 50–67 years old and this range is relatively younger than AD patients and needs to be expanded. Future studies will need to be conducted comparing NDE targets in normal aging and age-related cognitive impairment.

To further probe the NDE contents for potential targets, the Pulliam lab performed mass spectrometry on NDEs from 7 subjects, 5 uninfected controls (3 age-matched to the 2 HIV-infected subjects, 30–37 years old) and 2 older subjects, 57 and 66 years old, All samples were from subjects with normal cognition. NDEs were isolated using ExoQuick-Ultra and L1CAM monoclonal antibody immunoprecipitation as described previously (Sun *et al*, 2017). The method was modified to eliminate possible soluble contaminants by washing the NDE-resin complex with 1X PBS 5 times. Proteins were extracted from NDEs using M-PER lysis buffer without additive serum albumin or protease inhibitors. Proteins were trypsin digested and loaded onto a C18 column. Samples were analyzed on a nanoAcquity UPLC® coupled Xevo G2 QTof system. Mass spectrometry data were acquired using MassLynx 4.1 with positive polarity mode. Data were processed using Protein Lynx Global Server and viewed in Scaffold software. The databank used was constructed from the known sequence of the protein of interest plus other random proteins and common contaminants. Searches were performed with trypsin specificity and allowed for three missed cleavages. Possible structure modifications included for consideration were methionine oxidation and carbamidomethylation of cysteine. Proteins with 100% probability existing in at least two samples were reported. Several neuron-associated targets were identified (Table 1), not only confirming the neuronal enrichment of the exosomes but identifying potential targets to measure in NDE from impaired and unimpaired subjects. There were a number of familiar neuronal markers including APP, L1CAM, NCAM, neprilysin and NFL. The remainder are associated with synapse organization, synaptic signaling, cognition, neurogenesis and extracellular matrix. All of these proteins are potential biomarkers that may be further pursued in HAND, AD and other neurodegenerative disorders.

NDEs in Alzheimer’s Disease (AD).

The Kapogiannis lab has conducted a series of case-control studies that generated a set of NDE candidate biomarkers for AD. To maximize the potential for discovering differences in

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these studies, cases with high probability AD (i.e., low CSF Aβ42, high CSF tau and/or p181-tau) were matched to controls. The first study of NDE focused on the main pathogenic proteins measuring total tau, p181-tau, and Aβ42. Levels of these biomarkers, except for total tau, were higher in NDE from AD compared to control subjects achieving high classification accuracy; moreover, in a few cases with available samples, levels were already abnormal in preclinical AD (Fiandaca *et al*, 2015). Since then, other groups have shown increases of these markers in the progression from mild cognitive impairment (MCI) to dementia (Winston *et al*, 2016) and in Down syndrome (Hamlett *et al*, 2016), as well as low levels with normal aging (Abner *et al*, 2016). Next, we focused on other important pathways implicated in AD pathogenesis measuring key mediators. We found that NDE from AD cases show Ser/Tyr phosphorylations of the insulin receptor substrate 1 (IRS1) suggesting insulin resistance (Kapogiannis *et al*, 2015), elevated levels of lysosomal enzymes (cathepsin D, LC3) and ubiquitin suggesting lysosomal dysfunction (Goetzl *et al*, 2015b), decreased levels of cellular survival factors (REST) suggesting impaired cellular stress responses (Goetzl *et al*, 2015a); and decreased levels of synaptic proteins suggesting synaptic degeneration (Fig. 2) (Goetzl *et al*, 2018a; Goetzl *et al*, 2016). Most of these markers did not track disease severity and were not associated with cognitive performance, except perhaps for some synaptic proteins (Goetzl *et al*, 2018a; Goetzl *et al*, 2016).

In a recent Methods paper, the Kapogiannis lab showed that L1CAM exosomes are enriched for numerous neuronal markers including p181-tau, neuron-specific enolase, microtubule associated protein 2 (MAP2), neuron-specific class III β-tubulin (TuJ1), p35, NF-L, brain derived neurotrophic factor (BDNF), proBDNF, neuronal cell adhesion molecules NCAM and L1CAM; the degree of enrichment compared to total plasma exosomes and/or exosomes of variable cellular origin isolated by targeting CD81 ranged from 1.6-fold to 5.6-fold depending on the marker (Mustapic *et al*, 2017). Moving forward the use of exosome counts determined by NTA (as in (Eitan *et al*, 2017)) might gradually replace measurements of CD81 (as in (Fiandaca *et al*, 2015; Kapogiannis *et al*, 2015) unless another exosome marker, such as ALIX, could accurately reflect exosome counts . Normalizing to exosome counts is laborious, but CD81 may be present in only a fraction of plasma EVs and not particularly enriched in exosomes as opposed to microvesicles (Kowal *et al*, 2016). When studying different pathologies, exosome subsets in younger versus older individuals, normalization should be evaluated at the start and not assumed to be the same for all cohorts. If a tetraspanins or other exosome marker is to be used for normalization, there should be good correlation with counts.

As an alternative approach to mass spectrometry or targeted ELISAs to probe the NDE contents, the Kapogiannis lab used a multiplex proteomics technology (Fig. 3). Specifically, we used the Olink® Proteomics Biomarker Discovery Neurology panel (Olink® Proteomics, Boston, MA). The Neurology panel is a commercially available multiplex immunoassay enabling analysis of 92 neurology-related protein biomarkers using Proximity Extension Assay (PEA) technology. PEA is based on incubation of antibody pairs labeled with oligonucleotides and antigen of interest. Binding of the antibody pairs to the target brings oligonucleotide sequences in close proximity resulting in the formation of a PCR target sequence by a proximity-dependent DNA polymerization event. The newly formed sequence is quantified using standard real-time PCR where detection and sample analysis is performed

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by high-throughput real-time PCR analysis using the Fluidigm® BioMark™ HD System. Quality control and normalization of the samples was performed using Olink® NPX Manager software. From four plasma samples, we isolated total exosomes, NDEs and exosomes immunoprecipitated using antibody isotype control (IgG2a) and sent them to Olink for quantification blindly. NPX values were expressed as a log 2 scale where higher NPX values correspond to higher protein concentrations. Proteins with NPX values below a Lowest Limit of Detection threshold were excluded from further analysis. To visualize and present data we created a heatmap using ComplexHeatmap Bioconductor package (Fig. 3) (Gu *et al*, 2016). To interpret results, we surveyed and cross-referenced information in the Olink® and the Human Protein Atlas (https://www.proteinatlas.org/) websites and PubMed search results. Results show that a number of proteins known to be expressed in neurons (Alpha-2-MRAP, BMP-4, CPA2, CRTAM, DDR1, EZR, GCP5, IL12, LAT, LAYN, MANF, NMNAT1, NRP2, PRTG, RSPO1, SCARA5, SCARB2, sFRP-3, SKR3, TNFRSF21, VWC2) or even enriched for in neurons compared to other tissues (NTRK2, NTRK3, PLXNB3, THY1) were higher in concentration in NDEs, when compared to either total exosomes or IgG2a$^+$ exosomes. In addition, some proteins not expressed in neurons (FcRL2, MATN3, N-CDase, SIGLEC1) had a much lower concentration in NDEs compared to total and IgG2a$^+$ exosomes. Alongside this strong evidence for neuronal origin enrichment, there was also some evidence that particular NDEs may originate in non-neuronal tissues: a few proteins (CLEC1B, CLM-6, SMOC2) not yet shown to be expressed in neurons were nevertheless highly expressed in NDEs.

Although NDEs show consistently higher markers of proteins reasonably specific to neurons compared to total plasma exosomes (see (Mustapic *et al.*, 2017), L1CAM is also expressed in low levels in other tissues types (see https://www.proteinatlas.org/ENSG00000198910-L1CAM/tissue) and thus it is possible that a proportion of the signal detected may be of non-neuronal origin as suggested by our Olink results that show that a small number of proteins not previously shown to be expressed in neurons are present in NDEs.

Discussion

We have briefly discussed the use of NDEs as the source of biomarkers for neurodegeneration associated with cognitive impairment in HAND and AD. Examining neuronal damage can be performed in many other conditions where neuronal death is occurring, such as TBI (Gill *et al*, 2018), stroke, and other neurodegenerative diseases. Likewise, we have just begun to query the cargo of these exosomes (Mustapic *et al*, 2017) for a wide range of intracellular signaling molecules involved in multiple cascades that can be detected by immune-blot protein arrays in high levels of NDE and may constitute disease biomarkers (Mustapic *et al*, 2017)). Identifying multiple targets in exosomes is presently labor intensive, expensive and material-limited. For protein analysis, mass spectrometry is expensive, time consuming and inconsistent among individual samples. While multiple targets can be identified, follow-up protein analysis is necessary to determine differences between groups and some targets are below detection of ELISA assays and more sensitive procedures need to be developed (such as SIMOA®). Multiple target verification is expensive and together these steps require abundant sample. Multiplex arrays such as Olink® use small sample volume and multiple target panels. Although these techniques can

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identify low levels of proteins within samples and there is potential to identify previously unknown targets, as a survey tool, there are also limits of pre-selected panel composition, determining absolute protein concentrations and cost per patient.

Our studies to date have been confined to proteins, largely leaving mRNAs and miRNAs left to be explored. The goal would be to identify targets or pathways that are affected early by neurodegeneration or an age-related condition or from specific pathogenic effectors such as a virus. There are many advantages to looking at NDE cargo in neurodegeneration. Blood is easily accessible and the NDE contents will likely reflect neuronal damage in “real time”. Neurodegeneration across different pathological conditions may result in common pathways that may be targeted by experimental treatment strategies. The path forward for turning the promise of NDEs into a clinically useful tool includes further optimizing the isolation method by leveraging novel technologies, standardizing it and scaling it up for reproducible isolation of NDEs from large numbers of samples. Likewise, a panel of neurological targets could be created to evaluate the development and extent of damage and if treatment is available, the possible recovery of neuronal function. Moreover, recently, we demonstrated that it is possible to isolate exosomes from a specific neuronal subpopulation (Goetzl *et al*, 2018b). Given the roles of specific neuronal populations in neurodegenerative and neuropsychiatric disorders (for instance, dopaminergic neurons in Parkinson’s disease), we hypothesize that exosomes ostensibly originating from these neurons (for instance, dopaminergic ones) may represent a platform with even higher sensitivity and specificity for detecting molecular changes pertaining to the emergence of these disorders. All these developments have the potential to revolutionize biomarker discovery in neurological disorders and lead to a wide range of clinical applications.

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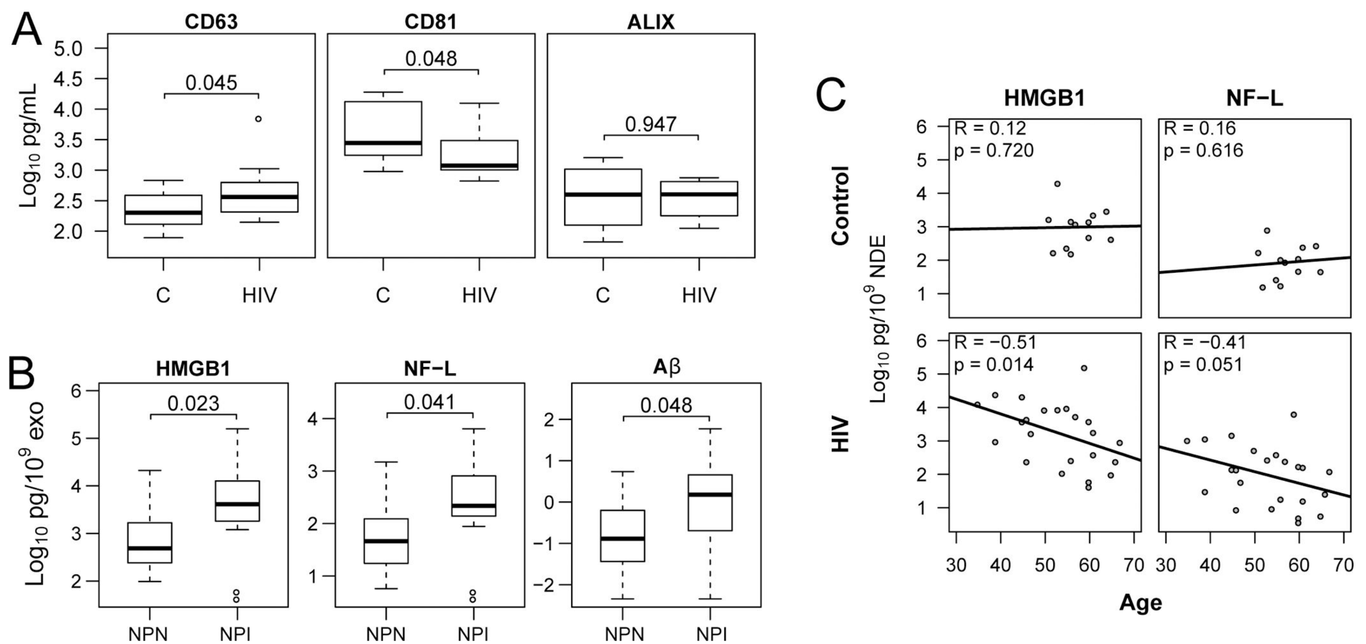
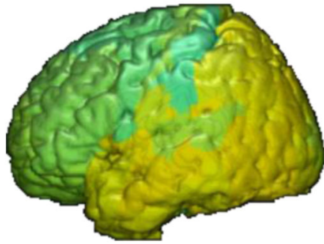
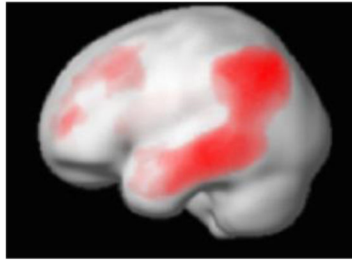


Figure 1: Protein expression and correlation of NDE biomarkers

from control (N=12) and HIV-infected individuals (N=23). (**A**) Tetraspanins CD63, CD81 and ALIX measured by ELISA in NDE. NDE from controls (C) and HIV-infected subjects (HIV) significantly differed in CD63 and CD81 levels. ALIX levels were not statistically different from NDE from controls and HIV infection. (**B**) Levels of proteins associated with neuronal dysfunction were assayed by ELISA from control subjects who were neuropsychologically normal (NPN, N=21) and neuropsychologically impaired (NPI, N=14) subjects that included 11 HIV-infected and 3 uninfected control subjects (Sun *et al*, 2017). HMGB1, high mobility group box1; NF-L, neurofilament-light; Aβ, amyloid β. (Adapted from (Sun *et al*, 2017)) **(C)** Aging and a decrease in NDE targets of HIV-infected subjects. Aging correlated with significant decreases in HMGB1 and NF-L in NDE from HIV-infected individuals. No effect was observed in NDE from controls. HIV-infected subjects had a wider age range (35–67 years) than the controls (51–65). Spearman correlation was used. (Adapted from (Sun *et al*, 2017))





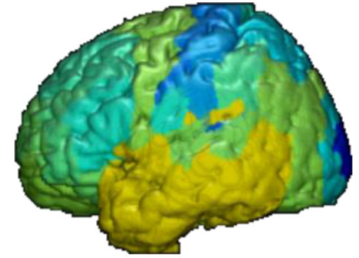


Figure 2: Protein targets in NDEs as biomarkers for AD.

Potential protein biomarkers are grouped by their relevance to different aspects of AD pathogenesis: Aβ Pathology and Synaptic changes (since Aβ directly induces synaptic pathology); Glucose Hypometabolism / Insulin Resistance (with a focus in insulin and downstream signaling cascades); and Tau Pathology / Neurodegeneration (including deficient neurotrophic factors). These targets were identified in referenced discovery studies by Kapogiannis, Goetzl, et al. and may be increased or decreased in AD patients compared to controls.

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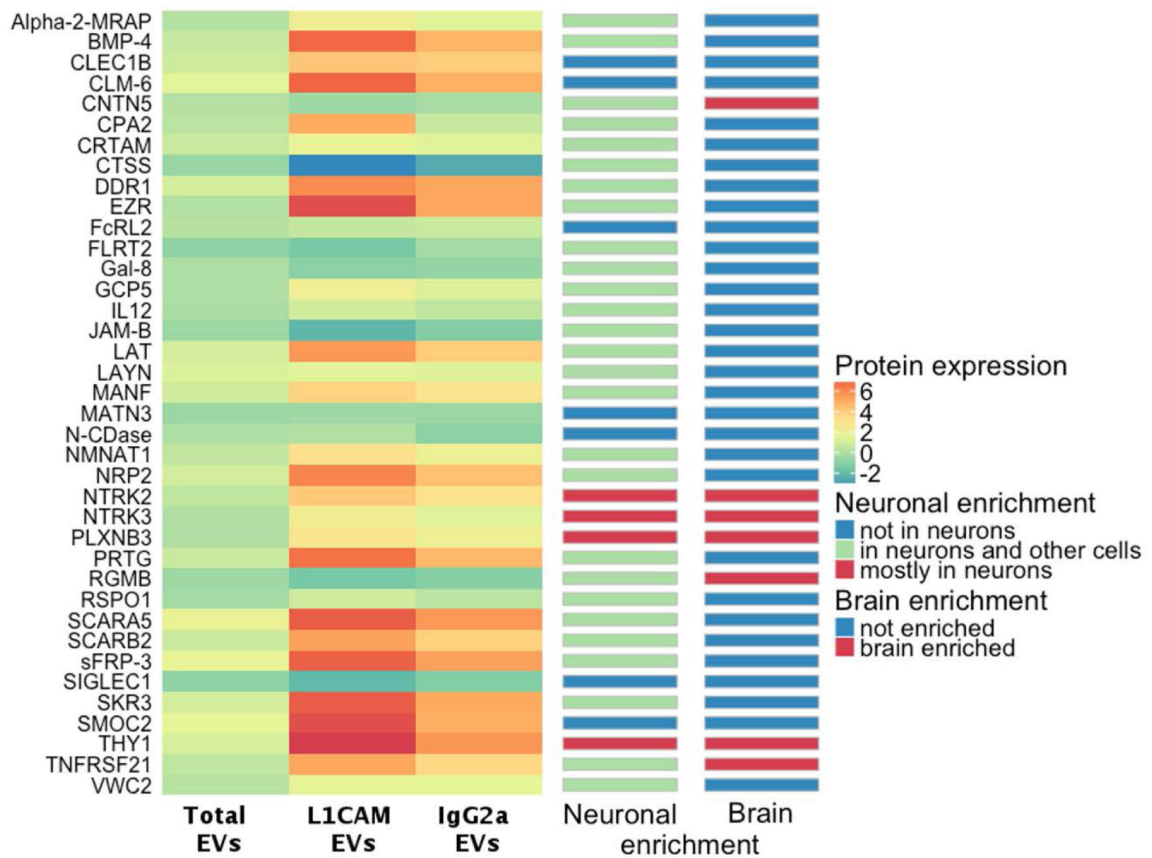

Figure 3: Heatmap of differential protein expression from EVs.

Heatmap showing log-transformed expression results (NPX values) for proteins measured in three distinct exosome populations (total, immunoprecipitated by anti-L1CAM and immunoprecipitated by anti-IgG2a) with the Olink Biomarker Discovery Neurology panel. Only proteins above the Lowest Limit of Detection are included. To help interpret results the two columns on the right indicate whether these proteins have previously been shown to be present or enriched in neurons (red - previously shown to be enriched in neurons compared to other tissues; green – previously shown to be expressed in neurons, but also in other cells; and blue – not previously shown to be expressed in neurons) and brain (red - previously shown to be enriched in brain tissue; blue - not previously shown to be expressed in brain tissue) according to Human Protein Atlas (https://www.proteinatlas.org/)and PubMed search results.

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Table 1

Neuronal proteins found in NDEs using Mass Spectrometry

Protein Name	Symbol
Amyloid-beta A4 precursor protein-binding proteins	APBA3, APBB1, APBB2
Neural cell adhesion molecule L1-like protein	CHL1 (L1CAM2)
Glutamate receptor ionotropic, NMDA 2D	GRIN2D
Neural cell adhesion molecule L1	L1CAM
Neprilysin	MME
Neuron navigators	NAV1, NAV2, NAV3
Neural cell adhesion molecules	NCAM1, NCAM2
Neurofilament light polypeptide	NEFL
Neurite extension and migration factor	NEXMIF
Neuroligin-4, Y-linked	NLGN4Y
Neurexin-1	NRXN1
Neurabin-1	PPP1R9A
Sodium channel proteins	SCN1A, SCN2A, SCN4A, SCN9A
Tenascin-R	TNR